

## Separable Domains Define Target Cell Specificities of an RTX Hemolysin from *Actinobacillus pleuropneumoniae*

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Received 30 May 1991/Accepted 23 October 1991

The leukotoxin (LktA) from *Pasteurella haemolytica* and the hemolysin (AppA) from *Actinobacillus pleuropneumoniae* are members of a highly conserved family of cytolytic proteins produced by gram-negative bacteria. Despite the extensive homology between these gene products, LktA is specific for ruminant leukocytes while AppA, like other hemolysins, lyses erythrocytes and a variety of nucleated cells, including ruminant leukocytes. Both proteins require activation facilitated by the product of an accessory repeat toxin (RTX) C gene for optimal biological activity. We have constructed six genes encoding hybrid toxins by recombining domains of *lktA* and *appA* and have examined the target cell specificities of the resulting hybrid proteins. Our results indicate that the leukocytic potential of AppA, like that of LktA, maps to the C-terminal half of the protein and is physically separable from the region specifying erythrocyte lysis. As a consequence, we were able to construct an RTX toxin capable of lysing erythrocytes but not leukocytes. The specificity of one hybrid was found to be dependent upon the RTX C gene used for activation. With *appC* activation, this hybrid toxin lysed both erythrocytes and leukocytes, while *lktC* activation produced a toxin which could attack only leukocytes. This is the first demonstration that the specificity of an RTX toxin can be determined by the process of C-mediated activation.

A number of pathogenic gram-negative bacteria secrete high-molecular-weight (100,000 to 110,000) calcium-dependent cytolytic proteins which are immunologically and genetically related to the alpha-hemolysin (HlyA) of *Escherichia coli* (7, 11, 17, 18, 21, 23–26). These toxins have been designated the repeat toxin (RTX) family on the basis of a series of glycine-aspartic acid-rich nonapeptide repeats found in the carboxyl-terminal third of the toxin protein (33). The genetic determinants for the secreted RTX toxins consist of four genes: A, the structural gene for toxin protein; C, which is required for activation of the toxin prior to secretion; and BD, which are necessary for the process of secretion. The four RTX genes are typically found in a single transcriptional unit, *CABD*, and are expressed from a common promoter located upstream of the C gene (19, 34, 38). In *Actinobacillus pleuropneumoniae*, however, the CA and BD gene pairs are unlinked and appear to have been derived from distinct RTX determinants (8).

The RTX toxins have been divided into two functionally distinct classes: the hemolysins, which lyse erythrocytes in addition to a variety of nucleated cell types, and the leukotoxins, which are lethal only to leukocytes. Within the leukotoxin class, species specificity is also observed, since the toxin produced by *Actinobacillus actinomycetemcomitans* attacks only primate leukocytes (31), while that of *Pasteurella haemolytica* is specific for ruminant leukocytes (30). The molecular basis of the species and cell type specificities of the RTX toxins is unknown. Comparisons of the predicted amino acid sequences derived from the two cloned leukotoxin genes and the cloned hemolysin genes from *E. coli* and *A. pleuropneumoniae* have not revealed

regions of homology which might account for the differing specificities of the two RTX classes (23). This analysis has identified two domains common to all known RTX toxins. One lies in the N-terminal half of the toxin protein and consists of four hydrophobic regions which may be involved in forming channels in target membranes (28). The second consists of a variable number (9 to 14) of the nonapeptide repeats which are believed to constitute a calcium-binding domain (4, 27). It is generally accepted that calcium is required for the biological activity of these toxins (3, 10, 27). Genetic experiments have demonstrated the existence of a third domain at the extreme carboxyl terminus of the RTX toxins (13, 14, 16, 22). This domain functions as a signal which is recognized by the RTX BD-dependent secretion pathway to direct transport of the toxin protein across the bacterial envelope. Despite the fact that the carboxyl-terminal domains of the RTX toxins are quite divergent in their amino acid sequences, the cloned BD genes appear to be interchangeable with respect to secretion of the RTX toxins from *E. coli* (5, 15).

Until recently, no function had been ascribed to the central region of the RTX proteins between the hydrophobic and nonapeptide repeat domains which constitutes 40% of the molecule. Recently, Forestier and Welch have demonstrated that a monoclonal antibody which recognizes only the activated form of HlyA binds to an epitope which maps just amino proximal to the beginning of the repeat domain (12). The existence of this antibody suggests that the HlyC-mediated activation of HlyA involves covalent modification of the HlyA protein. This undefined modification is essential for the binding of HlyA to erythrocytes (4). The HlyC protein has been shown to activate the AppA hemolysin from *A. pleuropneumoniae* (15) and the LktA leukotoxin

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from *P. haemolytica* (12). In contrast, the LktC protein was found to be incapable of activating HlyA (12), indicating that, unlike the *BD* genes, the *RTX C* genes are not interchangeable. Deletion analysis of the *lktA* gene has demonstrated that the region of LktA between the hydrophobic and nonapeptide repeat domains is responsible for its ability to bind productively to target leukocytes and is likely to determine its specificity (9, 10). Although the boundaries of this cell-binding domain have not been precisely delineated, this domain begins near codon 548 of *lktA* and may extend into or beyond the repeat region. Thus, evidence from both the HlyA and LktA systems suggests that the central third of the *RTX* toxins is required for binding to target cells and represents the fourth functional domain described for these proteins.

Given the high degree of similarity between the amino acid sequences and proposed domain structures of the *RTX* toxins, it should be possible to construct hybrid *RTX A* genes from which biologically active proteins are expressed. Such hybrids between an *RTX* hemolysin and an *RTX* leukotoxin gene would allow further definition of the domain(s) involved in toxin-cell interaction which is responsible for the differing specificities of the two *RTX* classes. Toward this end, we have constructed a series of hybrids between the *appA* gene of *A. pleuropneumoniae* and the *lktA* gene of *P. haemolytica*, which are nearly the same length (956 and 953 codons, respectively) and share 64% identity at the amino acid level (7, 23). We have chosen these genes in order to maximize the likelihood of obtaining functional hybrids. The biological activities and C dependence of the hybrids reveal new features of the internal organization of the *RTX A* genes and the process of their activation by *RTX C* proteins.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The *E. coli* host TB1 (20) was used for the preparation of lysates containing the wild-type and hybrid toxins employed in this study. Bacteria were grown in Luria-Bertani medium (10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter) supplemented with ampicillin (50 µg/ml) and/or kanamycin (50 µg/ml) when necessary. The bovine lymphoma cell line BL3 was grown in Leibovitz L-15 medium with glutamine (GIBCO Laboratories) containing 2.5% colostrum-free bovine serum (GIBCO Laboratories) and 2.5% calf serum (GIBCO Laboratories).

**Plasmid manipulations.** All plasmids used in this study were derived from the *lktCA* clone pYFC19 (6) and the *appCA* clone pYFC37 (7). The *appA* gene contains unique *HindIII* and *Clal* sites at nucleotides 1489 (Lys-497) and 2284 (Ile-762), respectively. The *lktA* gene contains a unique *Clal* site at nucleotide 2302 (Ile-768), and a *HindIII* site was created at nucleotide 1477 (Lys-493) by site-directed mutagenesis. The required single-base change does not alter the amino acid sequence of the LktA protein. By using these restriction sites to divide the *A* genes into three domains, a total of six chimeric toxin genes were generated by exchanging homologous restriction fragments between the appropriate plasmid pairs. Each of the initial constructs and the two parental plasmids had either *appC* or *lktC* upstream of the *A* gene. To construct clones with defective or deleted *C* genes, the *appC* genes were removed by digestion with *EcoRI* (which cuts in the vector polylinker) and *BclI* (which cleaves in the intergenic region between *appC* and *appA*). The

DNAs were then blunt ended with T4 DNA polymerase and recircularized by ligation in the presence of an *EcoRI* linker to regenerate an *EcoRI* site upstream of the *A* genes. The *lktC* genes were removed by digestion with *EcoRI* (cutting in the vector polylinker) and *BglII*, which cleaves at nucleotide 346 of the *lktC* gene. The DNAs were then treated as described above for the removal of the *appC* genes. Deletion alleles of the hybrids LAA, LAL, and LLA (see below) were generated by removing the *NaeI* fragment encoding residues 34 to 378 of the hybrid proteins. In all cases, the *A* genes are transcribed from the *lac* promoter of pHG165, which has a copy number of 20 to 30 per cell (32).

The *appC* and *lktC* genes from pYFC37 and pYFC19 were subcloned into the vector pAC18, which is a p15A replicon carrying a Kan<sup>r</sup> determinant and which has a copy number of 5 to 10 per cell (2). This vector is compatible with the vector pHG165, which is used to carry the parental and hybrid *A* genes. For *appC*, the 759-bp *EcoRI-BclI* fragment of pYFC37 was cloned into the *EcoRI* and *BamHI* sites of pAC18. For *lktC*, the 685-bp *EcoRI-NaeI* fragment from pYFC19 was cloned into the *EcoRI* and *SmaI* sites of pAC18. In both cases, the *C* genes are transcribed from the *lac* promoter of pAC18.

**Preparation of native and hybrid toxin proteins for bioassay.** Although the parental and hybrid toxins are secreted from *E. coli* strains expressing *RTX BD* genes, this process is inefficient, with the majority of the toxin activity remaining intracellular. Consequently, assays for biological activity were performed with lysates of *E. coli* expressing the desired *CA* gene pairs in the absence of *BD* gene function. *E. coli* cells containing the desired plasmids or plasmid pairs were grown overnight and then diluted in Luria-Bertani medium containing the appropriate antibiotics the following morning. At an optical density of 2.0 at 550 nm, cells were harvested by centrifugation, resuspended in 3.5 ml of calcium saline (10 mM Tris, 10 mM CaCl<sub>2</sub>, 0.8% NaCl [pH 7.4]), and disrupted by passage through a French pressure cell. The lysates were centrifuged at 100,000 × *g* for 1 h at 5°C to remove particulate material, and the supernatants were stored frozen prior to assay for hemolytic and leukotoxic activities.

**Assay for toxin activity.** Leukotoxin activity was measured as previously described (10). BL3 cells were washed and resuspended at a concentration of 2 × 10<sup>6</sup> to 3 × 10<sup>6</sup> cells per ml in Hanks balanced salt solution (HBSS) (0.8% NaCl, 0.04% KCl, 0.1% glucose, 2.5 mM CaCl<sub>2</sub>, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.4]). Aliquots of toxin were added to 1 ml of the cell suspension and incubated for 1 h at 37°C. Cells were then collected by centrifugation, resuspended in HBSS containing 0.05% neutral red, and incubated an additional hour at 37°C. The cells were then washed twice with HBSS and lysed with 500 µl of Nonidet P-40 buffer (10 mM Tris HCl [pH 7.5], 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation at 13,000 × *g* for 5 min, the supernatant containing the released neutral red was diluted in 0.5 ml of 0.1 M acetic acid, and the dye concentration was determined by measuring the A<sub>545</sub>. All results are expressed as percentages of the dye taken up by cells which had not been exposed to toxin. In this way, dose-response curves were generated for toxin-containing and control lysates. These data, in conjunction with the intensity of the toxin band on Western blots (immunoblots), allow the relative specific activities of the hybrids and parental toxins to be compared.

To determine hemolytic activity, aliquots of toxin were added to 1 ml of a suspension of bovine erythrocytes (3 × 10<sup>7</sup>

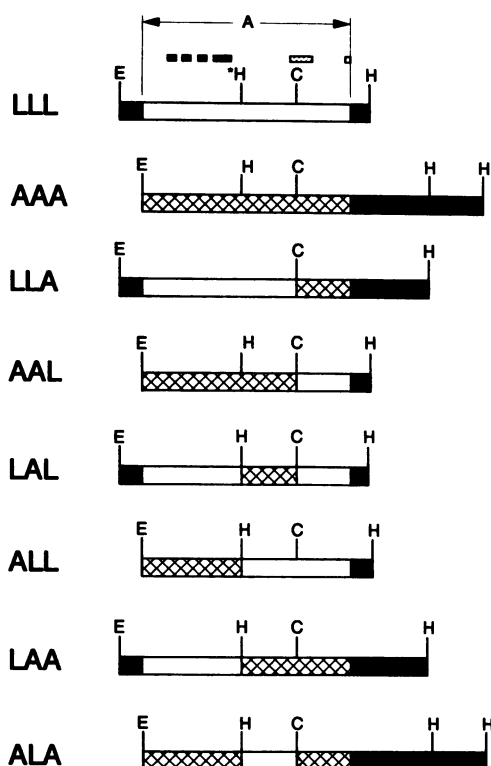


FIG. 1. Structures of the hemolysin-leukotoxin hybrid genes. The solid, hatched, and open boxes above the parental leukotoxin, LLL, show the locations of regions encoding the potential membrane-spanning region, the calcium-binding repeat domain, and the secretion signal, respectively. For the toxin genes, the open boxes represent sequences derived from the *Pasteurella* leukotoxin and the crosshatched boxes represent sequences from the *Actinobacillus* hemolysin. The solid boxes flanking the toxin structural genes represent incomplete copies of the parental *C* and *B* genes derived from the starting plasmids pYFC19 and pYFC37.

to  $4 \times 10^7$ /ml) in HBSS adjusted to 10 mM  $\text{CaCl}_2$ . After incubation for 1 h at  $37^\circ\text{C}$ , unlysed cells and erythrocyte ghosts were pelleted by centrifugation, and the amount of hemoglobin in the supernatant was determined by measuring the  $A_{540}$ . All results are expressed as percentages of the maximal hemoglobin released by lysing cells with Triton X-100.

**SDS-PAGE and Western blotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described by Altman et al. (1). Immunoreactive proteins were detected after transfer to nitrocellulose (35) with a mixture of bovine antileukotoxin (6) and swine antihemolysin (7) as the first antibody and a mixture of alkaline phosphatase-conjugated anti-bovine immunoglobulin G and anti-swine immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

## RESULTS

**Construction and characterization of the hybrid toxins.** The *appA* gene can be divided into three domains by using landmark restriction sites, as follows: domain I, from the start codon to the *Hind*III site at codon 497; domain II, from codon 498 to the *Cla*I site at codon 762 between nonapeptide repeats 5 and 6; and domain III, from codon 763 to the

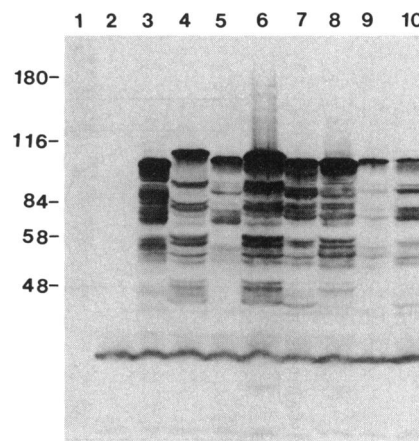


FIG. 2. Western blot analysis of the hybrid toxins. Lysates containing the *P. haemolytica* leukotoxin and the *A. pleuropneumoniae* hemolysin are shown in lanes 3 and 4. The remaining samples are lysates containing LLA (lane 5), AAL (lane 6), ALL (lane 7), LAL (lane 8), LAA (lane 9), and ALA (lane 10). Lane 2 is a control lysate from the *E. coli* host, TB1, which does not contain an RTX A gene. An equal amount of lysate protein was loaded in each lane. Immunoreactive bands were detected as described in Materials and Methods. The apparent molecular weights of prestained standards from Sigma Chemical Company are shown on the left (in thousands).

carboxyl-terminal codon 956 (Fig. 1). A *Hind*III site was created by a silent single-base change at the homologous site in *lktA* (codon 493), making it possible to divide this gene in the same way (Fig. 1). The domain II-III junction of *lktA* occurs between nonapeptide repeats 6 and 7. Each of the three domains can be assigned distinct and separable functions. Domain I contains the putative membrane-insertion sequences required for target cell lysis by both AppA and LktA (4, 26). Deletion analysis of *lktA* suggests that domain II of this toxin is responsible for target cell (leukocyte) specificity (10). Similar studies indicate that domain II of the AppA hemolysin is involved in binding to leukocytes (36). Domain III contains the carboxyl-terminal secretion signal common to the RTX family (13, 14, 16, 22). By exchanging these domains, six hybrid genes were constructed (Fig. 1). For simplicity, each toxin used in this study is represented by a three-letter acronym with A (for AppA) or L (for LktA) to identify the source of each domain. Thus, AAA and LLL are the parental toxins, while ALA is a hybrid with domains I and III from AppA and domain II from LktA. Since the *Cla*I site defining the boundary for domains II and III is not at an equivalent site in the parental genes, the exact number of repeats in the hybrid toxins varies. The parental toxins, ALL, and LAA have 9 repeats, hybrids AAL and LAL have 8 repeats, and hybrids LLA and ALA have 10 repeats.

The parental genes and each of the hybrids were placed under the transcriptional control of the *lac* promoter present on the vector pHG165. Translation of the resulting mRNAs relies on the translational start signals present in domain I. Western blot analysis (Fig. 2) indicates that the parental and hybrid LLA, LAL, AAL, and ALL toxin proteins were recovered in approximately equal amounts; hybrids LAA and ALA were recovered in 5- to 10fold-lower amounts. Although the parental toxins differ in length by only 3 amino acid residues and differ in predicted  $M_r$  by only 500 (AppA, 102,500; LktA, 102,000), the AppA protein has an apparent molecular mass 3 to 4 kDa greater than that of LktA, as



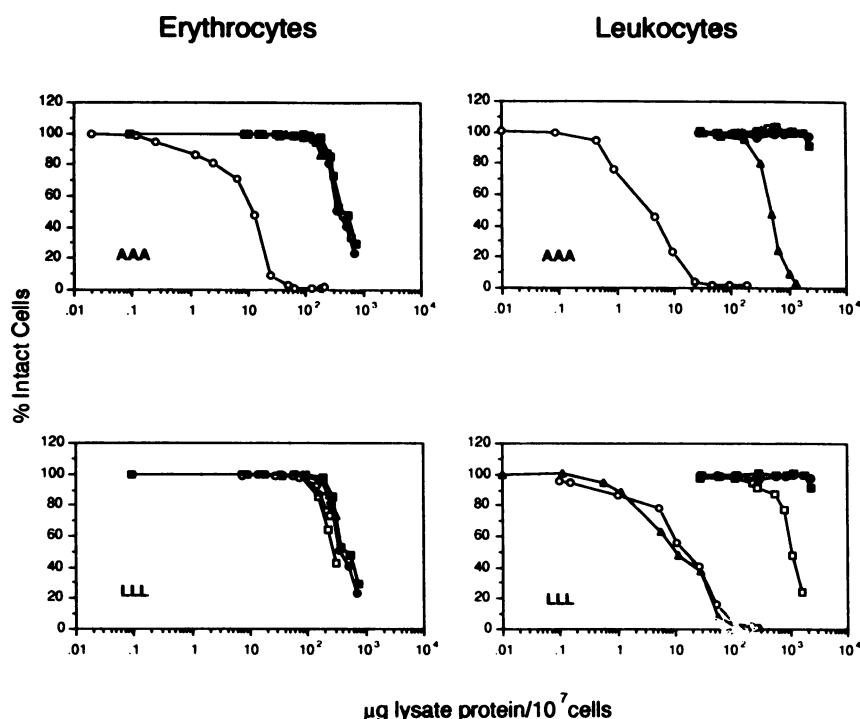


FIG. 3. Activation of the parental toxins by cognate and noncognate C proteins. Lysates were prepared from *E. coli* strains carrying the desired plasmids and assayed for lytic activity against bovine BL3 leukemia cells or fresh bovine erythrocytes as described in Materials and Methods. Each toxin protein was assayed without activation and with either AppC- or LktC-mediated activation. The nonspecific lytic activity of *E. coli* lysates was determined with lysates prepared from cells harboring pHG165 and the pAC18 clone carrying either *appC* or *lktC*. Symbols: □, toxin; △, toxin and LktC; ○, toxin and AppC; ■, pHG165 and LktC; ●, pHG165 and AppC.

determined by SDS-PAGE (Fig. 2). Each of the hybrid toxins was found to migrate between the two parental proteins, suggesting that this anomaly is not uniquely determined by one of the three domains defined in this study.

**Activation of the wild-type toxins by heterologous C genes.** Since it was not known whether the *appC* and *lktC* gene products were functionally equivalent, it was necessary to test each for the ability to activate the parental toxins, AppA and LktA. As the data in Fig. 3 indicate, the *appC* gene was capable of activating both toxins without alteration of target cell specificities. In contrast, the *lktC* gene product could only marginally activate AppA, giving toxin titers approxi-

mately 0.5% of that observed with AppC-activated AppA against leukocytes (Fig. 3; Table 1). Because of the higher background activity associated with the hemolysin assay, it was not possible to determine whether LktC-activated AppA displayed a similar, low hemolytic activity (nonspecific lysis of erythrocytes by moderate amounts of cleared *E. coli* lysates has been previously reported [37]). Lysates from *E. coli* expressing *lktA* in the absence of RTX C gene function were found to exhibit leukotoxin activity which was low (1% of that observed with LktC activation) but reproducibly above background levels (Fig. 3; Table 1). In contrast, AppA protein synthesized in the absence of AppC appeared to be lytically inactive (showing less than 0.1% of the activity observed with AppC activation).

**Novel phenotypes of the hybrid toxins.** Surprisingly, recombinants LAL and AAL were found to have a target cell specificity which differed from that of either of the parental toxins. Upon activation by AppC, both hybrids were found to lyse erythrocytes (Table 1) but had no detectable activity against leukocytes (Table 1). Neither protein could be activated by LktC (Table 1). To reflect this new specificity, we designate these two hybrids erythrocyte-specific toxins to distinguish them from the hemolysins, which lyse erythrocytes and leukocytes, and the leukotoxins, which are leukocyte specific.

Hybrid ALL also displayed an unexpected phenotype. When expressed in *trans* to *appC*, the toxin protein was found to lyse both erythrocytes and leukocytes, as does the parental AAA (Table 1). However, when activation was mediated by LktC, ALL was strongly leukotoxic but not erythrolytic (Table 1), as is the case with LktA. This is the

Toxin	Hemolysin with:		Leukotoxin with:	
	<i>appC</i>	<i>lktC</i>	<i>appC</i>	<i>lktC</i>
LLL	— <sup>b</sup>	—	20	10
AAA	10	—	5	500
LLA	—	—	—	—
LAA	30	—	70	—
LAL	40	—	—	—
AAL	20	—	—	—
ALL	100	—	30	5
ALA	60	—	500	—

<sup>a</sup> Data are expressed as the amount (micrograms) of lysate protein required for 50% lysis ( $L_{50}$ ) of the input cells ( $10^7$ ) after a 1-h incubation at 37°C. The  $L_{50}$ s are derived from titration curves similar to those shown in Fig. 3.  
<sup>b</sup> No activity above background levels. For erythrocyte lysis, the background level is typically 300 to 500 µg; for leukocyte lysis, the background level is >4,000 µg.

first indication that the target cell specificity of an RTX toxin might be determined by the identity of the C protein responsible for its activation. ALL was the only hybrid which could be activated by the *lktC* gene product. Hybrid LAA was found to be biologically indistinguishable from AppA (AAA), with lytic activity toward both erythrocytes and leukocytes only when activated by AppC (Table 1). Hybrid ALA was similar to AppA except that the ratio of its lytic activities toward leukocytes and erythrocytes was significantly less than that of AppA (Table 1). Only one hybrid, LLA, had no detectable activity when expressed alone or in *trans* to either *appC* or *lktC* (Table 1).

**Agglutinating phenotype of the hybrid toxins.** We have previously reported that the proteins expressed from certain in-frame *lktA* deletion alleles are capable of agglutinating bovine leukocytes in an LktC- and  $\text{Ca}^{2+}$ -dependent fashion (10). This agglutinating activity is seen only with deletions which are restricted to the region encoding the hydrophobic regions in the 5' half of the gene. Recently, we have found that similarly deleted *appA* alleles also give rise to proteins which agglutinate leukocytes (but not erythrocytes) in the presence of  $\text{Ca}^{2+}$  (36). Thus, the carboxyl-terminal halves of both the LktA and AppA proteins constitute autonomous leukocyte-agglutinating domains whose interactions with target cells can be investigated in the absence of cell lysis. In order to determine whether the differing abilities of hybrids LAA, LAL, and LLA to lyse BL3 cells reflect the functional statuses of their leukocyte-agglutinating domains, we deleted the entire LktA-derived hydrophobic region of domain I (codons 34 to 378) from these three hybrid alleles. The identical deletion in *lktA* has been shown to yield a protein which agglutinates, but does not lyse, bovine leukocytes (10). The hybrid genes containing this deletion were then expressed in *trans* to either *appC* or *lktC* and assessed for the ability to agglutinate BL3 cells. The deletion protein from LAA was found to agglutinate leukocytes only after *appC* activation (Fig. 4). Since its parent was lytic toward leukocytes only after *appC* activation, the ability of this hybrid to lyse leukocytes correlates with the ability of the carboxyl-terminal half of the protein to agglutinate this cell type. The deletion proteins from LAL and LLA could not agglutinate leukocytes after either *appC* or *lktC* activation (Fig. 4), suggesting that they contain a defective leukocyte-agglutinating domain. This is consistent with the inability of either parent hybrid protein to lyse leukocytes.

## DISCUSSION

Several studies have indicated that the HlyA protein is devoid of hemolytic activity when expressed in an *hlyC* *E. coli* host (12, 29). On the basis of these findings, it has been widely assumed that the biological activity of all other RTX proteins would also require modification by their cognate C gene products. However, in an earlier report, Forestier and Welch (12) showed that *E. coli* strains secreting the unmodified LktA protein exhibited leukotoxicity levels greater than background levels (see Table 1 in reference 12). In the present study, we have found that the LktA protein which accumulates intracellularly in a host without RTX CBD function has an activity which is roughly 0.5 to 1.0% of that observed for the same toxin synthesized in the presence of an RTX C protein. One interpretation of this observation is that LktA has an intrinsic lytic activity independent of C activation. This would imply that C activation merely increases the specific lytic activity of, or stabilizes, LktA.

Alternatively, this might indicate that there is a low level of LktA activation in the absence of C function. In this case, either the host provides a weak C-like activity or the RTX C proteins normally facilitate a modification process which involves host functions. These possibilities can be distinguished by a genetic analysis of the process of RTX A-protein activation.

While the AppC protein is able to activate both AppA and LktA, the LktC protein can only weakly activate the non-cognate toxin, AppA. Such nonreciprocal behavior has previously been reported for the HlyC-LktC and HlyA-LktA systems (12). In light of the ability of AppC to activate LktA, it is not surprising that AppC is capable of activating five of the six hybrid toxins employed in this study. The one exception, LLA, cannot be functionally activated by either C protein. The only hybrid toxin activated by LktC has the structure ALL, in which the C-terminal 460 residues are derived from LktA. This may indicate that the junction between domains II and III lies in a region of the protein which is critical for recognition by LktC. When regions flanking this junction are derived from AppA, activation by LktC is not possible. The hybrid ALL is also peculiar in that its specificity is determined by the nature of the C protein used for activation. When coexpressed with *appC*, the ALL protein is both erythrolitic and leukotoxic. However, activation by LktC produces a leukotoxin without detectable erythrolitic activity. This is the first instance in which the specificity of an RTX toxin has been shown to depend upon the mode of its activation. We propose that while AppC and LktC function similarly, they mediate modifications of the A protein which differ in number or location along the A polypeptide chain.

With the exception of LLA, all of the hybrids displayed erythrolitic activity. This suggests that there is sufficient information present in either domain I or domain II of AppA to endow a hemolysin-leukotoxin hybrid with erythrolitic activity (for example, both ALL and LAL are hemolytic proteins). In contrast, only hybrids in which domains II and III are both derived from the same parent were found to exhibit leukotoxic activity comparable to that of LktA and AppA. The inability of AAL and LAL to lyse leukocytes is probably not due to a failure of C activation, since these hybrids were completely inactive when expressed in the absence of a C gene but became active erythrocytes when coexpressed with *appC*. Furthermore, the AppC-activated hybrid ALA exhibited erythrolitic and leukotoxic activities approximately 25 and 1%, respectively, of those seen with AppA. Thus, C activation can have differential effects on the leukotoxic and erythrolitic potentials of an RTX toxin, a phenomenon which may explain the phenotype of hybrids AAL and LAL. The erythrocytes AAL and LAL also differ from the parental toxins in that each contains one fewer Gly-Asp repeat. These repeats constitute the calcium-binding region of the RTX toxins (4) and are essential for their lytic activity (3, 10). It is possible that the altered repeat region is responsible for the absence of leukotoxic activity for AAL and LAL. If this is true, the effect of the altered repeat region does not extend to the erythrolitic activity of these hybrids, which is easily assayed and remains calcium dependent.

We have previously shown that the carboxyl-terminal halves of AppA (36) and LktA (10) are capable of agglutinating BL3 cells. When the lytic domains of hybrids LAL (erythrocyte), LAA (hemolysin), and LLA (nonlytic) were removed, only the deletion protein derived from LAA displayed the agglutinating phenotype characteristic of similar



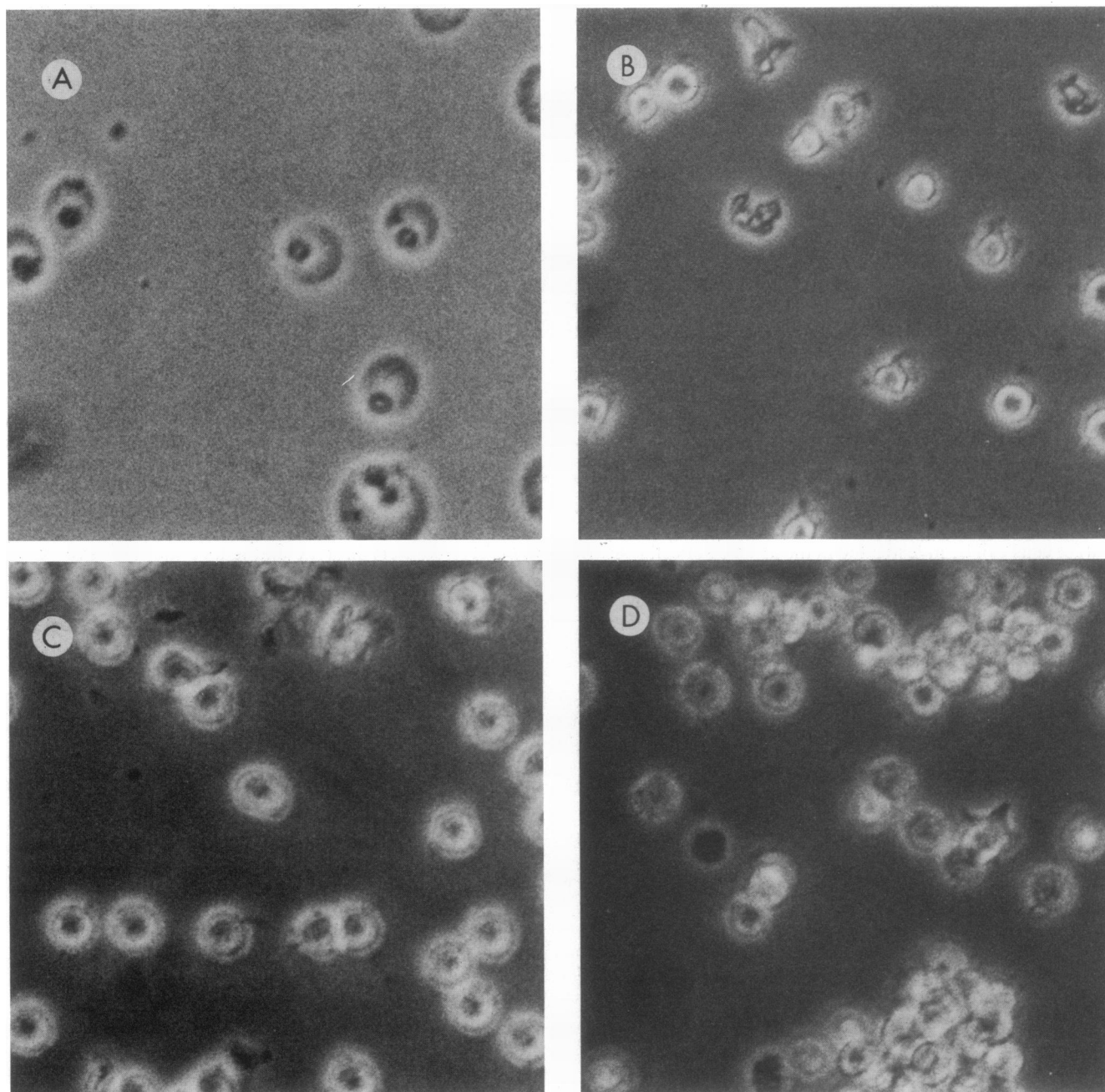


FIG. 4. Hybrid LAA contains a leukocyte agglutination domain. Lysates were prepared from *E. coli* strains expressing the *appC* gene and either the wild-type *appA* gene or the domain I deletion alleles of LAA, LAL, or LLA. The lysates were incubated with BL3 leukemia cells under standard assay conditions and examined for lysis or agglutination by phase-contrast microscopy as previously described (10). (A) AppC-activated AppA; (B through D) AppC-activated deletions of LLA, LAL, and LAA, respectively. Magnification, ca.  $\times 150$ .

deletion alleles of the parental toxins. Thus, the ability of the C-terminal halves of AppA, LktA, and the hybrids described in this study to agglutinate BL3 cells appears to be correlated with the ability of these proteins to lyse leukocytes and is unrelated to erythrolytic potential. Taken together, our findings imply that (i) the hemolytic and leukotoxic potentials of AppA are determined by different, but perhaps overlapping, regions of toxin protein which respond differently to AppC and LktC activation, (ii) the erythrolytic activity of AppA is specified by functionally independent or redundant elements found in both domains I and II of the

protein, and (iii) the leukotoxic activity of LktA and AppA requires specific interactions between domains II and III of the toxins which occur only when both domains are derived from the same parent.

#### ACKNOWLEDGMENTS

We are grateful to Lydia McCartney for administrative assistance, M. Craig Miller and Roger Livingston for technical assistance, and the Biomedical Communications Office of the College of Medicine for photographic services.

This work was supported by an AUF grant from the Texas A&M University System (to R.Y.) and USDA grant 88-37266-3926 (to R.Y. and D.K.S.).

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